Applications of droplet-based microfluidics to identify genetic mechanisms behind stress responses in bacterial pathogens

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ABSTRACT

The primary bacterial targets for most antibiotics are well known. To survive the stress of an antibiotic a bacterium must decrease the antibiotic to target binding ratio to escape from harmful effects. This can occur through a number of different functions including down-regulation of the target, mutation of the binding site on the target, and decreasing the intake or increasing the efflux of the antibiotic. However, it is becoming more evident that an antibiotic stress response influences more than just the primary target, and that a wave of secondary responses can be triggered throughout the bacterium. As a result resistance mutations may arise in genes that are indirectly affected by the initial interaction between the antibiotic and target. These indirect responses have been found to be associated with metabolism, regulation, cell division, oxidative stress, and other critical pathways. One technique recently developed in our lab, called transposon insertion sequencing (Tn-seq), can be used to further understand the complexity of these indirect responses by profiling growth rates (fitness) of mutants at a genome-wide level. However, Tn-seq is normally performed with large libraries of pooled mutants and thus it remains unclear how this may influence fitness of some independent mutants that may be compensated by others in the population. Additionally, since the original method has only utilized planktonic culture, it is also not clear how higher order bacterial structures, such as biofilms or microcolonies, influence bacterial fitness. To better understand the dynamics of pooled versus individual mutant culture, as well as the effect of community structure in microcolony development on the influence of fitness, we adapted a droplet microfluidics-based technique to encapsulate and culture single mutants. We were able to successfully encapsulate at least 7 different species of bacterial pathogens, including

Streptococcus pneumoniae, and culture them planktonically, or as microcolonies, in either monodisperse liquid or agarose droplets. These experiments, however, raised an important challenge: the DNA yield from one encapsulation experiment is insufficient to generate samples for sequencing by means of the traditional Tn-seq method. This led us to develop a novel Tn-seq DNA library preparation method, which is able to generate functional Tn-seq library molecules from picogram amounts of DNA. This method is not ideal yet because fitness data generated through the new method currently does not correlate well with data from traditional Tn-seq library preparation. However, we have identified one major culprit that should be easily solvable. We expect by modifying the binding site of the primer used for linear amplification of transposon ends that the new preparation method will be able recapitulate results from the traditional Illumina preparation method for Tn-seq. This will enable us to prepare robust Tn-seq samples from very small amounts of DNA in order to probe stress responses in single mutants as well as in microcolonies in a high-throughput manner.

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INTRODUCTION

The problem.

Antibiotic resistant bacteria are a major health concern with an infectious burden causing over 2 million illnesses and 23,000 deaths per year in the United States alone[1]. This overwhelming number of bacterial infections has led to an estimated $35 billion a year in direct medical costs and lost economic productivity[1,2]. There are three major approaches that have been applied throughout the past 60 years to try to curb antibiotic resistance and its associated cost: **1) vaccine development; 2) phenotypic screens to identify compounds with antibiotic properties; 3) bioinformatic analysis of genome sequences for rational drug targets and subsequent chemical library screens.** Vaccine development was a tremendous breakthrough, allowing for the reduction of major bacterial infections from pathogens such as *Neisseria meningitides, Vibrio cholera, Corynebacterium diphtheria,* and *Streptococcus pneumoniae*[3]. Although vaccines have significantly reduced the number of total bacterial infections, they are often not 100% efficacious or fully protective against all pathogenic strains within a species[4–7]. Vaccines were eventually supplemented with antibiotics, which were identified through phenotypic screens during the 1950s and 1960s in what is termed as the "Golden Age of Drug Discovery". Systematic methods were used to test if soil microorganisms harbored antibiotic properties against common bacterial pathogens. Many of our current antibiotics originate from these screens, but these methods have been mostly abandoned because of diminishing returns due to the exhaustive identification of antibiotics with a high discovery frequency. Re-discovery of the same compounds makes it extremely difficult to identify novel antimicrobials that are present at extremely low frequency[8]. The third

approach to curb antibiotic resistance takes advantage of the tens-of-thousands of fully sequenced eubacterial genomes that have been generated since the mid-1990s[9]. Bioinformatic identification of conserved bacterial genes, which were tested for growth essentiality, produced hundreds of potential drug targets that were screened for interactions against large chemical compound libraries. Although this approach has produced several promising leads, such as FabI-directed antibiotics[10], it has had limited success compared to phenotypic screens. Large pharmaceutical companies who had adopted large chemical screens have since mostly abandoned the method[9,11]. Due to the limitations of the above applications we desperately need to find new methods for solving the problem of antibiotic resistance in bacteria. So where do we start?

The hypothesis.

We propose to temporarily zoom out and focus our attention on what actually makes a bacterium able to withstand and deal with the stress they experience when challenged with an antibiotic. How antibiotics cause bactericidal or bacteriostatic affects is much studied and vigorously debated. It is well known that antibiotics have a primary target, mainly DNA, RNA, or protein. Most of the major routes of escape from antibiotics have been shown to minimize the interaction between the drug and the bacterium through three general mechanisms: 1) reduced influx and increased efflux of the antibiotic, 2) mutation at the binding site of the primary target, and 3) targeted break-down of the antibiotic. However, clinical strains often show multiple genotypic variations that contribute to resistance, but these alterations are located in genes whose primarily role is not resistance, and are instead involved in in fundamental bacterial processes. Additionally,

data from network biology approaches have shown that the relationship between a bacterium and an antibiotic reaches far beyond its direct-target. Instead an antibiotic triggers a complex, multi-factorial process that may begin with the physical interaction between the drug and its target but quickly propagates into the involvement of a variety of processes that can include regulation, metabolism and/or energy generation. **These data have lead us to hypothesize that a novel antimicrobial strategy could originate from a comprehensive understanding of which bacterial genes are involved in dealing with a particular antibiotic stress. Genes that become important in the presence of an antibiotic are thus potentiating genes that could function as secondary targets that make existing antibiotics more powerful. Moreover, these stress-responsive genes could also indicate where adaptation is most likely to occur in the genome. We thus believe that identification and an in depth analysis of these antibiotic stress-related genes is critical in our fight against antibiotic resistant bacteria.**

The model system.

In this study we ultimately seek to generate genome-wide stress-response profiles, or gene-antibiotic interaction maps (GAIM), for the bacterial pathogen *Streptococcus pneumoniae.* With over 800,000 deaths worldwide, half a million of those being children under the age of 5, *S. pneumoniae* poses a considerable health threat[12]. Pneumococcal diseases such as pneumonia, bacteremia, otitis media, and meningitis, also present a serious health risk for the elderly, the immune-compromised, and those people receiving organ transplants[13]. One of the best preventative measures we have for pneumococcal

disease is a conjugate vaccine, Prevnar 13, which is only ~60% efficacious[14]. Moreover, it only protects against 13 out of more than 90 pneumococcal serotypes[7] and there is a concern for serotype replacement[15], in which non-vaccine serotypes increase in asymptomatic carriage and disease-causing cases. Because of this lack of full protection, antibiotics that can broadly target a species remain a necessary defense against *S. pneumoniae* infections. However, as with so many bacterial pathogens the number of singly and multiply drug resistant *S. pneumonia* cases is steadily increasing[16–18], exacerbating the problem of treating pathogenic infections.

The challenge.

To accurately identify genome-wide genetic factors involved in an antibiotic stress response we first propose to use the systems tool Transposon insertion sequencing (Tn-seq), which takes advantage of massively parallel sequencing to accurately determine genotype-phenotype relationships on a genome wide scale[19]. We have previously shown the broad applicability of Tn-seq by 1) determining fitness cost of all non-essential genes and possibly essential genes, in several clinically relevant strains of *S. pneumoniae*[20,21], 2) generating high-confidence gene-condition interaction maps for a number of typical challenges that pathogenic *S. pneumoniae* encounter during infection and treatment[21], and 3) proving the functionality of the method in not only *in-vitro* conditions, but also *in-vivo* mouse models[22]. However, one limitation of Tn-seq is that mutant libraries are grown in batch culture, thereby possibly masking mutants that are outcompeted for available resources as well as allowing "cheaters" to survive by taking advantage of overall population fitness. Therefore the current Tn-seq technique may not be entirely

representative of true mutant fitness, or more importantly, be completely lacking in some important mutant data. Additionally, sequencing sample-preparation requires micrograms of DNA, which we discovered to be problematic for the experiments proposed below, where only nanograms are available.

The solution.

We address the limitations of Tn-seq in this study by taking advantage of droplet microfluidics to encapsulate and culture complex mutant transposon libraries of bacterial cells. Encapsulation can be performed in such a way that each droplet contains a single mutant, and each droplet has its own independent environment. As a result individual bacterium within the library can no longer interact with each other, the confounding factor of 'population fitness' is removed from the experiment and the individual mutants must rely on their own genotype-phenotype characteristics for survival. In addition, the droplets allow us to keep the total culture volume quite low, saving on cost and time that is a marked improvement compared to the next best compartmentalization tool, which is the microtiter plate.

Experimental design.

In this study we setout to develop both a droplet-microfluidics system and a low input Illumina DNA library preparation for Tn-seq with the purpose of: 1) studying how the fitness of individual bacteria are influenced by culture in a dynamic population, and 2) studying the impact the microcolony mode of growth has on bacterial fitness. We first adapt and characterize a microfluidics system which produced monodisperse water-in-oil

droplets at rates upwards of 1000 droplets/sec. Single *S. pneumonia* cells can be encapsulated in droplets and cultured over time, which does not affect the growth profile compared to standard liquid growth. In addition to planktonic growth in liquid droplets, we develop a complementary assay for microcolony-like growth in agarose droplets. The ability to culture microcolonies is critical since most antibiotic studies are performed using planktonic cultures even though most naturally occurring bacteria survive in higher-order structures, such as microcolonies and biofilms. We show some basic characteristics of microcolony-like growth in agarose droplets across three different species of bacteria including *S. pneumoniae*, *Acinetobacter baumannii*, and *Streptomyces roseosporus*. Finally a low input Illumina DNA library method for Tn-seq was tested against nanogram and picogram amounts of DNA and then processed through an Illumina next-generation sequencing platform. The resulting sequence data is analyzed using scripts developed in-house and the number of sequencing reads is used to generate fitness data for different low DNA input amounts and correlated to the traditional Tn-seq preparation method.

METHODS

Microfluidic Device fabrication

Microfluidics devices are developed by applying poly(dimethyl siloxane) (PDMS) to a microfabricated silicon mold. The silicon mold is a generous gift from the lab of David A. Weitz at Harvard. The pattern for generating the mold is designed using autoCAD software (Fig. 1). A PDMS mold is generated by mixing PDMS and curing agent (Dow Corning, Sylgard 184) in a 10:1 ratio and mixing vigorously with a fork. The mixture is added into the silicon mold and degassed with a vacuum for 20 min and cured at 65°C overnight. Cured PDMS is cut out of the mold with a scalpel and the non-patterned side of the slab covered with scotch tape. A biopsy punch (0.75mm – Shoney Scientific) is used to create ports for the tubing (PE-2 tubing – Intramedic). The PDMS slab is then bonded to a glass slide (Corning – 2947, 75x50mm) at a cleanroom facility. First the glass is cleaned with acetone and methanol in a sonicator bath while the PDMS is cleaned with methanol, and then all materials are thoroughly dried with filtered nitrogen gas. The channel side of the PDMS slab and the glass slide are treated with plasma (400sccm flow; 400 watts; 60 sec) using a faraday barrel screen. Plasma treated surfaces are quickly brought into contact and held together using even pressure across the entire PDMS surface by sandwiching the chip between two large glass plates and pressing down on a table. After 2-5 minutes of applying pressure the device is placed at 65°C for 10 minutes to complete the bonding.

Microfluidic Device treatment and Droplet production

Before droplet production the devices are treated with the water-repellent Aquapel

(Aquapel - item# 47100). Channels are first primed with fluorinated oil (Novec 7500 oil; 3M – cat# 98-0212-2928-5) by connecting a 1-ml syringe (Luer-Lok Tip; BD – ref# 309628) with an attached 27-G needle (BD – ref# 305109) to the oil inlet port using PE-2 tubing (Fig. 1.1). The channels are then primed with Aquapel using a 5ml SGE Gastight syringe (Fisher - SG-008760). The channels are incubated in Aquapel for 10-30 seconds before flushing with Novec 7500. Devices are used immediately or incubated overnight at 65°C and then stored in the dark for several weeks before use. Syringe pumps are used to push reagents into the microfluidic device to create droplets. First a 1-ml oil syringe is filled with 1.5% of PicoSurf 1 (Dolomite; 3200214) and attached to the oil inlet port (Fig. 1.1) using PE-2 tubing. The flow rate is set to 750 μl hr^{-1} and pushed through the device until the oil exits the droplet outlet port (Fig. 1.5), at which point PE-2 tubing is attached to the outlet port. Flow through is collected in a 1.5ml microcentrifuge tube. While the oil phase is still being pushed through, the aqueous phase is first prepared by filtration through a 0.22μm syringe filter and then filling a separate 1-ml syringe. PE-2 tubing is connected to aqueous syringe, primed with a 500 μl hr^{-1} flow rate, and then attached to the aqueous inlet port (Fig. 1.2). Droplet production is observed in real-time with a light microscope.

Bacteria strains, growth, and media

Experiments are performed using three *S. pneumoniae* strains: TIGR4 (NCBI Reference Sequence: NC_003028.3); Taiwan-19F (NC_012469.1); D39 (NC_003098.1), as well as two additional species: *Acinetobacter baumannii* (CP000521.1) and *Streptomyces roseosporus* (GCA_000156695.2). Except for Tn-seq experiments *S. pneumoniae* strains

are cultured statically in Todd Hewitt broth supplemented with yeast extract (THY) plus 5 µl/ml Oxyrase (Oxyrase, Inc) and catalase (Worthington Bio Corp LS001896), or on Sheep's blood agar plates at 37°C in a 5% CO_2 atmosphere. *A. baumannii* is cultured in Luria-Bertani (LB) broth or on LB agar at 37°C. *S. roseosporus* is cultured in LB broth or LB agar at 30°C.

Transposon library construction

Library construction using the mariner transposon Magellan6 is performed as previously described[19,23,24]. The transposon lacks transcriptional terminators allowing for read-through transcription, and additionally has stop codons in all three frames in either orientation to prevent aberrant translational products. Six independent transposon libraries are produced for each *S. pneumoniae* strain (TIGR4, Taiwan-19F, D39). Each library consists of approximately 10,000 total mutants.

Culture of Bacteria in droplets

Bacteria are cultured to exponential phase before diluting and encapsulating into either liquid or agarose droplets. For liquid droplet culture experiments in which growth curves are generated (i.e. Tn-seq libraries, antibiotic treatments), culture is performed in semi-defined minimal media (SDMM)[23] at pH 7.3 supplemented with 20 mM glucose. For Tn-seq, input samples are collected before encapsulation, and the sample is split for either standard pooled culture or droplet encapsulation. Standard pool culture is diluted to an initial OD_{600} of 0.003 (5million cells/ml), while droplet devices are seeded with a concentration of 50 million cells/ml. The standard conditions for producing liquid

droplets are 500 μl hr^{-1} aqueous flow rate and 750 μl hr^{-1} oil rate unless otherwise noted. Encapsulation is performed for 15 minutes for all liquid droplets experiments. Liquid droplets are cultured at 37°C and 5%CO_2 atmosphere unless otherwise noted. To produce growth curves from liquid droplet culture small fractions of the entire droplet culture are collected at different time points and broken open with 1H,1H,2H,2H-perfluoro-1-octanol (PFO; Sigma-Aldrich, cat. no. 370533) and subsequently plated on agar for live cell counts. At the end of culture a fraction of the droplets is observed under a Zeiss Axioplan 2 microscope and the remainder is used for genomic DNA extraction isolated using the DNeasy kit Blood and Tissue Kit (Qiagen – 69506) with modifications. To generate agarose droplets the entire droplet production system is placed in a 37°C room. A 1.5% agarose (Sigma Type IX-A, Ultra-low Gelling Temperature) mixture is made using growth media and then heated until dissolved. The agarose is then filter sterilized (0.22μm) after which the cells are added to the agarose solution. The same series of encapsulation steps are then taken as with production of liquid droplets, except that the standard flow rate for agarose droplets is 1000 μl hr^{-1} oil rate and 250 μl hr^{-1} aqueous rate. Droplets are then either cultured under the bacteria's most optimal growth temperature conditions, or placed below room temperature (<20°C) for at least 30 minutes to allow for agarose gelling.

DNA sample preparation and Illumina sequencing

Tn-seq DNA sample preparation is performed depending on high (>500ng) or low (<500ng) DNA input amounts. High DNA inputs amounts (>500ng) are prepared with a traditional Illumina preparation method for Tn-seq as previously described[20,22]. Low

DNA input amounts (<500ng) are prepared by the following method: A 5' biotinylated primer complementary to the transposon inverted repeat sequence (Table 1; Bio-TEG_P1_M6_MmeI_Short) is used to generate single-stranded DNA (ssDNA) using NEB Q5 high-fidelity polymerase in a 20µl reaction with 8% polyethylene glycol 8000 (PEG 8000) with the following incubation: 98°C for 2 min, followed by 50 cycles of 30s at 98°C, 5s at 53°C. ssDNA product is incubated with 20ul of Ampure XP beads (Beckman Coulter - A36880) for 20 minutes and washed three times in 70% EtOH. DNA is eluted off of the beads and resuspended in second strand synthesis buffer [1X random hexamers (Sigma 11277081001), 1X NEB2 buffer, 5 units NEB Klenow exo-, 0.5mM dNTP, 8% PEG 8000] and incubated for 30min at 37°C followed by 20min at 75°C. The sample is cleaned one more time on Ampure XP beads before being incubated with 20ul of streptavidin beads (ThermoFisher - Dynabeads MyOne Streptavidin C1, cat # 65001) at room temperature for 1hr. Streptavidin beads with bound DNA are washed twice with binding and washing (B&W) buffer (1M NaCl, 0.5mM EDTA, 5mM Tris, pH 8) followed with two washes in dH_2O. Streptavidin beads are resuspended in MmeI digestion buffer (2 units NEB MmeI enzyme, 50µM SAM, 1X CutSmart, 8% PEG 8000) and incubated for 2.5hrs at 37°C followed by 20min at 65°C. 1µl of alkaline phosphatase (NEB - M0290S Calf Intestinal, CIP) is added to beads and incubated for 1hr at 37°C. Adapters are made by mixing and equal volume of primers ADBC-F and ADBC-R (Table 1) that are each at 0.2 nM with 1mM Tris and then incubated with the following condition: 96^{o}C for 2 min followed by 20-24^{o}C for 10 min. Streptavidin beads are washed and added to adaptor ligation buffer (NEB 1X T4 ligation buffer, T4 DNA ligase, 8% PEG 8000) and 1µl of 6pM adaptor (Table 1) and incubated at 16°C overnight

followed by enzyme inactivation at 10min at 65°C. Streptavidin beads are washed and the library is finally amplified using NEB Q5 polymerase and a transposon-specific primer along with an adapter-specific primer (Table 1; P1M6 and AdptTnseq primers) using the following incubation: 98°C for 30sec, followed by 20-26 cycles of 20s at 98°C, 5s at 62°C, 5s at 72°C, followed by 20s at 72°C. PCR products are gel purified and sequenced on an Illumina NextSeq 500 series sequencer according to the manufacturers protocol.

Sequence analysis and Tn-seq fitness calculation

Sequence analysis is performed with a series of in-house scripts as previously described[20,22]. The fitness of a single mutant (W_i) is calculated by comparing the fold expansion of the mutant to the fold expansion of the population and is determined by the following equation as explained previously[20]:

$$W_i = \frac{\ln(N_i\,(t_2)\times d/N_i\,(t_1)\,)}{\ln\,((1-N_i\,(t_2))\times d/(1-N_i\,(t_1)))}$$

W_i takes into account the average fitness value across all insertions within one gene, and standard deviation as well as a weighted average allow for the determination the most significant data. Since fitness is calculated using the expansion factor of the population (d), W_i becomes independent of time, therefore allowing comparisons between different strains and conditions across different experiments. gDNA from mutant libraries in which we already had traditional Tn-seq fitness data were used to test the new library preparation explained above. In this way correlations of fitness values could be made between the previous data and the new preparations to assess quality and composition.

Additionally genes that had at least one insertion were compared between low input preparations and the traditional preparation.

RESULTS & DISCUSSION

I) Droplet Tn-seq workflow: Library culture in droplets, sequencing, and fitness calculations.

Significant progress was made in two critical parts of a new Tn-seq workflow (Fig. 2) which were: 1) characterizing the production of picoliter-sized droplets through a microfluidics device (Fig. 2a-b), and 2) a new Illumina sample library preparation method for low input amounts of DNA from Tn-seq libraries (Fig. 2c-e). To characterize the droplet-based microfluidics system an in vitro technique was used to generated a complex library of mutants using the Mariner Himar1 mini-transposon derivative *magellan6* as previously described[20]. The *magellan6* transposon inserts into TA sites and results in pooled libraries of mutants where each bacterium contains one insertion (Fig. 2a). Culturing of bacteria was performed in picoliter-sized droplets and compared to standard batch culture, which is normally used for Tn-seq[20] (Fig. 2b). The small droplet volumes generated by a microfluidics system allowed for the isolation and culture of single mutants from the pool, which must rely on their own phenotype-genotype characteristics for survival. Because of the small amounts of genomic DNA that were recovered from these droplets, we subsequently developed and validated a novel low input Illumina sample preparation method, which was performed on genomic DNA from three independent Tn-seq libraries from the *Streptococcus pneumoniae* strain TIGR4 (Fig. 2c-e).

II) Development and characterization of a droplet-based microfluidics system to generate liquid droplets.

One goal of this study was to evaluate how the growth of individual bacterial strains is influenced when grown in a mixed batch culture. The first step to address this question was to utilize an assay that separates out complex mixtures of bacteria. Droplet microfluidics has been utilized by other groups for single-cell analysis through the compartmentalization of individual bacteria[25–29]. These studies have been used to manipulate and study single cells in a variety of ways including transfection, directed evolution, gene expression analysis, RNA-sequencing, enzymatic activity, and genome analysis[30]. Besides the ability of droplet-microfluidics to separate a bacterial culture into single cells, we hypothesized that it would be highly complementary to Tn-seq, enabling high-throughput single-cell genomic analyses.

II-A) Monodispersity of liquid droplets.

First a microfluidics device (Fig. 1) was generated and tested for the droplet encapsulation of a liquid aqueous phase into an oil-surfactant mixture. It was critical that the droplets being produced were monodisperse, meaning that all the droplets are of uniform size. Monodispersity of droplets was essential to prevent biases in bacterial growth due to varied abundance of resources across each droplet. Microfluidic liquid encapsulation produced droplets with diameters that had an average coefficient of variation (CV) of 2.7%. This distribution is well within the acceptable range found in

other studies[28], which should not influence growth between different droplets simply due differences in volume.

II-B) Frequency of production, capacity, and stability of liquid droplets.

The production frequency and the size of the droplets that were produced were controlled by altering the flow rates of the oil and aqueous phases. The oil flow rate ranged between 625-1000 μl hr^{-1} and the aqueous flow rate ranged between 150-500 μl hr^{-1}. Since the speed and size of the droplets were controlled by the ratio of the oil flow rate to the aqueous flow rate we represent this value with an Oil:Aqueous flow ratio (OAR). The standard OAR used for a majority of the experiments included in this study was 1.5 OAR (750 μl hr^{-1} oil: 500 μl hr^{-1} aqueous), which generated droplets of 40.5 μm ± 1.1 μm in diameter or 35 pL ± 0.7 pL in volume. With this flow rate frequencies of approximately 4000 droplets/sec was generated, and in a standard encapsulation time of 15 minutes that produced approximately 3.5 x 10^6 droplets, which rivals frequencies produced by other groups[31]. Droplet production was tested across a range of flow rates and found an achievable OAR range of 1.25-6.67 (625–1000 μl hr^{-1} oil : 150-500 μl hr^{-1} aqueous) without device failure. This allowed the production of droplets that ranged in size from 34μm in diameter (20.5 pL) to 42μm in diameter (39 pL) (Fig. 3a-b). The average standard deviation in size of droplets for all tested flow rates was 1.3μm in diameter. The results for generating liquid droplets were highly reproducible and OAR correlated well with diameter of the droplets (R^2 = 0.82; Fig. 3c). Importantly, droplets were stable for several days at different growth temperatures including 37°C which is the optimal temperature for our model organism, *Streptococcus pneumoniae.* Additionally, droplets

were stable under slow shaking conditions (10-50rpm) over several days. Shaking is advantageous because droplets are encapsulated in fluorinated oil, which is gas permeable. Thus continuous shaking allows for even gas exchange, which permits the uniform growth of aerobic species that must be cultured non-statically.

III) Fine tuning of liquid droplet culture conditions for three different strains of *Streptococcus pneumoniae* in the absence and presence of an antibiotic.

After developing optimal conditions for generating monodisperse liquid droplets the culture of individual bacteria in pico-liter sized compartments was compared to that of standard batch culture. Batch culture represents a complex environment in which many different bacteria are able to interact with each other. One of the goals in this thesis was to develop an assay to determine how interactions among mutants in a population affect each other's behavior. To solve this problem we took a reductionist approach, where the problem (population of mutants) is split into its simplest parts (individual mutants) and each is studied independently.

III-A) Separation of a complex pool of cells into droplets based on a Poisson distribution.

A reductionist approach was applied to a population of cells by using droplet microfluidics to split every individual cell into an independent growth environment. Assuming that loading rate was constant we can predict the load occupancy of the droplets based on a Poisson distribution[32] (Fig. 4). If encapsulation is completely random than the probability of finding x number of cells per droplet can be predicted using the

equation $P(X = x) = e^{-\lambda}[\lambda^x/x!]$, where λ represents the mean number of cells per droplet volume. Therefore, using an initial mean number of 0.3 cells per volume ($7.5x10^6$ cell/ml) reduces the chances of getting 2 cells or more in one droplet to ~3%. At this concentration approximately 23% of the droplets will contain one cell and 74% will contain no cells. Thus, to empirically determine if a majority of our droplets that are occupied contain just a single cell we simply needed to determine what percentage of our droplets were empty. Loading the droplets with a culture that had an absorbance (OD_{600}) of 0.03 gave ranges of empty droplets from 85%-70% (Fig. 5) depending on the bacterial strain used and how vigorously the sample was vortexed before encapsulation. These results are reasonable considering that different strains have slightly different cell numbers at the same OD_{600} and that vortexing causes cellular chains of *S. pneumoniae* to be broken up resulting in an increase in the observed live cell concentration.

III-B) Growth profiles of 3 different *S. pneumoniae* strains in liquid droplets.

Next the culture rate of *S. pneumonia*e in droplets was compared to that of batch culture. For this growth was tested between three *S. pneumonia* strains (TIGR4, D39, and 19F) that differ in their relatedness (Fig. 6). Droplets were loaded with a concentration of approximately 7.5 x 10^6 cell/ml in a semi-defined minimal media (SDMM). Encapsulation was performed for 15 minutes resulting in a predicted total of 3.5 x 10^6 droplets of which 7.5 x 10^5 were predicted to be filled with a single cell. Cells were cultured at 37°C and samples were collected and plated on blood agar plates over a period of 8hrs. Growth curves revealed that strains did expand in droplets and that these growth profiles had similar trends to batch culture (Fig. 7). Further experiments showed

that bacteria cultured in droplets do lag somewhat in growth compared to batch culture and that a greater than 10-fold expansion can be achieved in batch culture (on average approximately 300 fold expansion) compared to that in droplets (on average approximately 30 fold expansion). This phenomenon is likely due to waste products, such as hydrogen peroxide, building up much more quickly in the droplets compared to the large diffusion space offered by batch culture. Growth differences between droplet and batch culture may also be due to differences in buildup of quorum sensing molecules[26].

III-C) Genomic DNA extraction from one droplet culture experiment.

Initial tests also found that the total amount of genomic DNA that could be extracted from one droplet experiment was approximately 10ng. This is a critical factor because we want to prepare this DNA sample for transposon sequencing and we discovered that yields this low make it difficult to successfully generate an Illumina sequencing library. One simple solution may be to encapsulate for longer times, which would generate greater numbers of encapsulated cells and therefore lead to a higher DNA yield. Although this solution is impractical considering the doubling time for *S. pneumoniae* (~35min) and the long encapsulation time that would be necessary (>15min). Cells that would be encapsulated at the very beginning of droplet making would have already have gone through nearly one doubling inside of the droplets compared to those encapsulated towards the end of the droplet making. This would generate a heterogeneous cell-growth state across droplets and skew true fitness values of independent mutants.

III-D) Growth profiles of *S. pneumoniae* in liquid droplets in the absence and presence of Penicillin.

S. pneumoniae cells were then cultured in droplets and compared to batch culture under a stress response. Penicillin G was chosen as an antibiotic stress due to its clinical application in the treatment of *S. pneumoniae* infections[33–35]. Also using a new approach to discover genetic factors associated with Penicillin stress would be advantageous considering the widespread resistance to this drug acquired by *S. pneumoniae*[36,37]. The *S. pneumoniae* strain TIGR4 (T4) was treated in the absence and presence of Penicillin (0.0165ug/ml – 0.2ug/ml) in both droplet and batch culture. It was observed that the antibiotic inhibitory range of T4 in the presence of Penicillin was similar between droplet and batch culture (Fig. 8). This was somewhat unexpected considering that *S. pneumoniae* already showed signs of inhibited growth in droplets demonstrated by the reduced expansion factor compared to batch culture in the absence of Penicillin. Then again Boedicker *et al.* and other groups have successfully established antibiotic susceptibility testing of bacteria in droplets[25], proving that antibiotic treatment in droplets should be representative of batch culture.

IV) Modification of the droplet-based microfluidics system to generate agarose droplets.

In addition to using a microfluidics system to generate monodisperse liquid droplets, conditions were also developed to produce monodisperse agarose droplets. Agarose droplets are appealing for a number of reasons including: 1) the ability to replenish nutrient resources for encapsulated cells[28], 2) providing encapsulated cells with a solid

matrix for adhesion[28,38], 3) creating double encapsulations including agarose-in-aqueous-in-oil (ag/aq/o) droplets or an agarose-in-agarose-in-oil (ag/ag/o) droplets, or 4) the ability to sort encapsulated cells by FACS without having to re-inject droplets into the device to create water-in-oil-in-water (w/o/w) droplets. While all of these applications could be used to study bacteria in interesting ways the primary purpose for producing the agarose droplet system was to generate an environment where bacteria could form microcolonies. This is critical because most current assays that study antibiotic stress do so exclusively with planktonic culture. By generating agarose droplets that were the same size as our liquid droplets we create a well-controlled experiment to compare planktonic versus microcolony growth. We describe the development of agarose droplet production and report on a few noteworthy phenotypes we observed. Future experiments will entail more in-depth characterization of microcolony development in agarose droplets such as growth rate and treatment with antibiotics.

IV-A) Monodispersity of agarose droplets.

Similar to the liquid droplets optimal encapsulation conditions were determined for agarose including the range of flow rates and the distribution of droplet sizes that could be generated. It was discovered that a crucial factor in the production of monodisperse agarose droplets was the careful control of temperature during encapsulation. The most consistent agarose droplet sizes were generated by using an Ultra-low melting temperature agarose in a 37°C environment. The constant 37°C temperature prevented the agarose from experiencing minor fluctuations in viscosity during encapsulation, therefore allowing consistent droplet formation. Although the gelling temperature of the

ultra-low melting agarose was below room temperature, performing the encapsulation at 22°C produced polydisperse droplets and device failure due to delamination of the PDMS device. It was discovered that the variability in agarose droplet size (CV = 7%) under multiple different flow rates was larger than that of liquid droplets. Although the agarose droplet size variance was larger than in liquid droplets the volumetric range still well within that found by other studies that have produced agarose droplets[29,38].

IV-B) Frequency of production and capacity of agarose droplets.

The operational range of OA flow ratios for agarose droplet production is 1.50-8 (750-1000 μl hr^{-1} oil rate; 125-500 μl hr^{-1} aqueous rate), which is similar to that of liquid droplets. There was a very poor linear correlation between OAR ratios and agarose droplet diameter (Fig. 9). This indicates that there are either critical, but yet to be identified, procedural factors that are changing from experiment to experiment, or that the relationship between flow rates and droplet diameters is not truly linear, which some studies have suggested[39]. Additional testing is required to find out the reason behind this variability. Since flow rate is a good predictor of droplet size, it should be controlled more carefully for agarose droplet production. The syringe pumps are not likely the issue because the same pumps are used for both liquid and agarose droplet production. Another critical factor for flow rates is the hydrophobic treatment of the channels prior to encapsulation. The exact residence time of the hydrophobic treatment is not known, so degradation of the hydrophobic interactions could be a likely reason behind agarose droplet size variation.

Additionally it was observed that the agarose droplet size was much smaller than that of liquid droplets under similar OAR conditions (21 μm - 34 μm in diameter or 5 pL - 20.5 pL in volume) (Fig. 9). This may be due to the extra drag caused by the agarose polymer against the sides of the channels. The drag would slow down the flow rate of the aqueous phase, causing the observed OAR ratio to be higher than expected which would produce smaller droplets. Efforts to try to increase the aqueous rates beyond the described thresholds to overcome this proposed drag resulted in device delamination and failure due to extreme pressures. If we could determine the factor that controls the variation in agarose droplet size from experiment to experiment, we could consistently generate similar sized agarose and liquid droplets (~35um or 22pL) with the current device. This would allow us to accurately compare planktonic and microcolony culture with the only variable being the agarose matrix, and with no other confounding variables, which would be a marked improvement over other types of microcolony assays[40,41].

The number of agarose droplets produced in a certain amount of time could not be determined due to the variation in the production. However it can be concluded that more consistent sized agarose droplets can be made with aqueous flow rates at or below 250 μl/hr. When the collective variances are averaged below or equal to an aqueous flow rate of 250 μl/hr the CV decreases from 7% to 5.8%, while flow rates above 250 μl/hr have a collective CV average of 8.9%. This indicates that monodisperse agarose droplets cannot be produced as quickly as liquid droplets in the same microfluidics device. It is also worth noting that droplets have been generated using different concentrations of agarose from 1-2%. However these concentrations have not been systematically tested to determine the overall affect on droplet generation.

V) General characteristics and notable phenotypes observed in microcolony development across different bacterial pathogens.

It is well documented that microcolony attachment and aggregation is the first step in biofilm formation[42,43]. Communal modes of growth, such as biofilms and microcolonies, are known to contribute to resistance against stresses such as antibiotics. However most antimicrobial studies are routinely performed using planktonic cells. Therefore there is a need to develop assays to address the impact of microcolony formation on the antimicrobial stress response of bacteria. Here we develop a microcolony-like growth assay in agarose droplets that is complementary to the liquid droplet assay described previously. The effects of growth in agarose droplets were tested across three bacterial species with different characteristics: *S. Pneumoniae* (gram positive), *Acinetobacter baumannii* (gram negative), and *Streptomyces roseosporus* (filamentous actinobacteria). First *S. Pneumoniae* was encapsulated as single cells or at high density in 1.5% agarose in THY media. Droplets were cultured at 37°C and 5% CO_2 and DIC images were taken at 4hrs and 20hrs (Fig. 10). When seeding droplets with single cells it was noticed that microcolonies approximately 10μm in size were produced after 4hrs. Microcolony formation was not observed in droplets seeded in a high density after 4hrs. Nutrient depravation, waste product build-up, or quorum sensing activity may account for this phenotype. After 20hrs of growth microcolonies that were produced in droplets seeded with single cells remained relatively intact while cells in the high-density droplets looked like they had undergone cell death. The most likely reason for cell death is hydrogen peroxide buildup, which is a well-known characteristic of *S. Pneumoniae,* which keeps it

from having a stable stationary phase. This still begs the question as to why the microcolonies remained visually intact even though hydrogen peroxide was also building up over a 20hr period. More tests will be needed to answer these questions. We did however determine basic growth requirements in agarose droplets for *S. pneumoniae,* which include a short culture time (4-6hrs) and would likely be more optimal if we removed the oil and refreshed the media after several hours.

A. baumannii was seeded as single cells or at high density into 1.5% agarose droplets in LB media. These droplets were then incubated at 37°C for 24hrs at which point DIC images were taken (Fig. 11). The oil was then removed from the droplets using PFO and the droplets were put into fresh LB. After 48hrs of total growth more DIC images were taken (Fig. 11). *A. baumannii* has much more robust and uniform microcolony growth compared to that of *S. pneumoniae.* Additionally hearty *A. baumannii* microcolonies are formed in droplets seeded with both single cells and at high density, albeit high-density droplets have microcolonies that are smaller in size compared to that of droplets seeded with single cells. This difference in microcolony size may have to do with nutrient limitations. To prove that active growth could be re-establish we removed the oil and put the agarose droplets into fresh media. The observation of microcolonies expanding to a larger size in the high density condition by 48hrs total growth compared to droplets in which oil was not removed (not shown) demonstrated that fresh nutrients could replace waste products inside droplets and trigger active growth (Fig. 11). Interestingly intact microcolonies could not be found in droplets that were initially seeded with single cells after 48hrs with oil removal and nutrient replacement. Instead only droplets that had tears in them were found. These torn droplets looked like they had at one point harbored

a microcolony. Those droplets that were seeded with many cells and therefore had many microcolonies in them remained intact while ones that were seeded at single cell density and had very few colonies tore through and escaped the confinement of the agarose. We therefore speculate that this "escape" phenomenon is density dependent. It is exciting to think this observation occurred due to a signaling molecule that controls microcolony dispersal. At higher density there may be more of this molecule around to signal the bacteria to prevent dispersal. When this molecule is lacking the bacteria may trigger twitching or swarming motility to escape the droplet; and there is evidence that extracellular conditions can strongly regulate motility in *A. baumannii*[44]. If identified this molecule could be taken advantage of to prematurely disperse microcolony structure of *A. baumannii,* which could theoretically make these bacteria more susceptible to antibiotics.

S. roseosporus is used here because it represents a dynamic group of filamentous soil bacteria that are responsible for a majority of our current antibiotics[45]. *S. roseosporus* itself produces the precursor to the novel antimicrobial Daptomycin[46]. Although the precursor to Daptomycin is not studied directly here we determined growth characteristics of *S. roseosporus* in agarose droplets that build the foundation for future studies of the precursor. First *S. roseosporus* spores were produced. This was necessary because *S. roseosporus* cells divide in long continuous filaments, which make it difficult to seed individual cells in separate droplets. Spores were used to seed droplets that contained different concentrations of agarose (0%, 1%, 1.5%, and 2%) and cultured at 30°C for 48hrs. Fascinatingly *S. roseosporus* filaments punctured through the oil layer at higher concentrations of agarose (Fig. 12). A likely explanation for this is that *S.*

roseosporus can push better off of the denser agarose matrix and can use the apical end of a single filament to penetrate through the oil. Although this explanation hypothetically makes sense it is still curious considering the seemingly opposing interactions that would occur between the oil and the cell membrane. Ultimately optimal growth conditions for culturing and retaining *S. roseosporus* in agarose droplets was found, which is seeding in 1% agarose and culturing at 30°C for 48hrs.

VI) Development and validation of a novel low-input Tn-seq DNA library preparation method.

A major drawback in the production of good quality DNA libraries from transposon insertion mutants is the need for high input amounts of DNA[20]. Therefore assays in which only a small amount of DNA is recovered, like single-cell genomics and in-vivo experiments, require further manipulation after the primary culture cycle in order to generate enough DNA for sequence preparation. In the case of Tn-seq these manipulations require either: 1) the continued proliferation of cells on solid media or, 2) an increase in the number of PCR cycles to amplify the final DNA library. Both of these methods can skew the data representing the distribution frequency of mutants in the population. Therefore a new method was establish to generate Tn-seq DNA libraries from low DNA inputs by enriching genomic DNA near transposon ends with a single stranded DNA PCR using a biotinylated primer. The DNA was then cleaned up with solid phase reverse immobilization (SPRI) beads and then bound to streptavidin beads for adaptor ligation (Fig. 2c-d).

VI-A) Enrichment of transposon ends using a biotinylated oligo.

Biotinylated oligos can be used for Pyrosequencing, SNP analysis, solid-phase DNA sequencing, and a number of other applications in which the strong streptavidin-biotin bond is used to immobilize or enrich DNA molecules. Biotinylated oligos have also been used to successfully generate single stranded DNA libraries from ancient or damaged DNA[47]. Biotinylated primers are used here to enrich transposon ends from DNA of a complex transposon mutant library. This technique has been utilized previously by Goodman et al. in a method called Insertion sequencing (INSeq)[48]. The lower limit of DNA input for this method is 500ng. Due to the limited amount of DNA recovered for some growth experiments, such as in-vivo colonization and the single-cell genomics assay described in this study, it was necessary to develop a low input DNA library preparation that was sensitive down to 10ng or less.

VI-B) PCR validation of library preparation using a combination of SPRI beads and streptavidin beads.

It was determined that the difficulty in enriching a genomic region using a biotinylated primer is due to excess unused primer. The unused biotinylated primer could bind streptavidin beads and caused unwanted PCR products. Once the unused primer was bound to the beads the unbiotinylated end was still able to ligate to a sequencing adaptor, which is a necessary step in library preparation. During library preparation this direct primer-adaptor ligation outcompeted the development of the true library and created a product that was ~20bp smaller than the true library size (Fig. 13a). By cleaning up the reaction with commercially available SPRI beads (Ampure XP beads) directly after the

enrichment with the biotinylated primer, excess biotinylated primer was removed to prevent the unwanted PCR product. Ampure bead removal of excess unused primer was verified by performing a library preparation in the absence and presence of genomic DNA from a transposon library (Fig. 13). In the absence of genomic DNA there were no amplified PCR products, while in the presence of genomic DNA a PCR product of the predicted library size is generated. Also the amount of DNA library that was amplified was dependent on the initial concentration of primer used in the first single stranded PCR reaction. Additionally since it was shown previously that using poly(ethylene glycol) 8000 (PEG) can increase the efficiency of enzymatic reactions[47] we decided to utilize this method for our low input library preparation. It was determined that more single stranded product could be produced from a 20 µl reaction with 8% PEG than in a 50 µl reaction in the absence of PEG with holding all other variables constant (data not shown). This is why 8% PEG was used in all enzymatic reactions during library preparation. Additionally, an advantage of using streptavidin beads during cleanup steps for the low input library is the removal of all nonspecific PCR products (Fig. 13). In the traditional preparation nonspecific products are produced at approximately 100bp and 30bp (Fig. 13, +ctl). These nonspecific products are presumably due to excess adapter, which is removed during wash steps for the new low input preparation.

VI-C) Validation of the low input Tn-seq DNA library preparation by massively-parallel sequencing.

To validate the low input DNA library preparation method three biological replicates of a Mariner transposon library produce in an unencapsulated version of TIGR4 (2394) were

processed. These libraries were cultured in SDMM in the presence of glucose. To rigorously evaluate the new low input preparation method the same genomic DNA was utilized to generate sequencing libraries using the new preparation method for the three input amounts (10ng, 1ng, and 100pg) and generate libraries using the traditional Tn-seq prep with 3µg of DNA[20].

PCR products of the correct size were generated from the new sequencing preparation method with all low input amounts and from the traditional Tn-seq preparation (data not shown). These samples were pooled, processed through the Illumina NextSeq sequencer according to the manufacturer's protocol, and the data analyzed according to a Tn-seq workflow previously described[20]. We were able to successfully extract the number of insertions per gene and generate fitness values from this data. After combining the data for all three libraries the quality of our traditional preparation was considered satisfactory because the number of insertions per gene correlates extremely well to a separate biological replicate experiment using the same library samples (Fig. 14a; R^2=0.99). Additionally the fitness values correlated well for a biological replicate (Fig. 14; R^2=0.74,). However there is no correlation between any of our new low input preparations and the traditional preparation for either number of insertions per gene or fitness (Fig. 14a-b). Additionally when comparing total number of insertions (or unique reads) across the genome the low input library preparation had on average 88% fewer reads then the traditional preparation. The reason behind this significant loss in robustness in the low input preparation is not due to data being thrown out during sequence analysis because 99.9% of all reads were kept after fastq filter and 95% of that data was properly demultiplexed. The reason behind the lack of correlation is thought to

be due to the binding site of the biotinylated primer from the initial single stranded PCR. The biotinylated primer is complementary to the inverted repeats found on the very ends of the transposon. Therefore the first nucleotide that is incorporated during the single stranded enrichment will be in the genomic DNA. This means that the single stranded DNA enrichment could be subjected to bias depending on which nucleotide is incorporated. PCR bias resulting from mixtures of complex templates has been described previously[49]. Goodman et al. avoid this bias by having the recognition site for single stranded PCR be approximately 20bp away from either end of the transposon[48]. In this way the first 20 or so nucleotides that are incorporated across all transposon insertion sites are exactly the same, which reduces PCR bias. This lack of diversity could also be due to a combination of sub-optimal thermal-cycling conditions and poor polymerase enzyme kinetics. To address this a number of different polymerases and thermal-cycling conditions will be tested to optimize the technique. Although very poor correlations were obtained when comparing fitness values between the new low input preparation and the traditional library preparation, we still managed to successfully produce an Illumina sequencing preparation technique for Tn-seq samples that works down to picogram amounts of DNA input. Success in the new sequencing preparation is represented by the number of genes found with insertions that overlap between the new preparation and the traditional preparation (Fig. 14c).

CONCLUSIONS

It is paramount to develop methods that are both accurate and dynamic in the information they can provide to us about a biological system. We first describe a method that could reveal the influence of bacterial population fitness on the fitness of individuals within that population. We also describe a method that could reveal the importance of bacterial microcolony development in fitness and dealing with a stress response. Significant progress has been made in developing and characterizing critical aspects for both of these methods. We can successfully generate monodisperse liquid and agarose droplets, in which we have optimized growth of several clinically relevant pathogens. Going forward it will be necessary to eliminate the variation in the generation of agarose droplets. We are also making strides to generate new microfluidic chips, which can generate larger droplets to allow for greater expansion of encapsulated bacteria. Another new chip design could allow us to produce alginate droplets as an alternative to agarose droplets. Alginate droplets would be more optimal because they are more biologically relevant, considering that some bacteria secrete alginate in biofilms. Additionally alginate droplets can be made at room temperature eliminating the need for a warm room and potential fluctuations in flow rates, which would reduce droplet size variation considerably. We have also generated a new Illumina sequencing preparation method that is sensitive down to sub-nanogram of DNA input. This is not only important to our new droplet Tn-seq method, which only produces nanograms of DNA, but also to other Tn-seq library culture methods that generate only small amounts of DNA such as *in-vivo* studies. We have identified a very likely source to the current bias generated in our low input sequence

preparation. Changing the recognition site of the primer for single stranded PCR enrichment should eliminate this problem.

FIGURES & TABLES

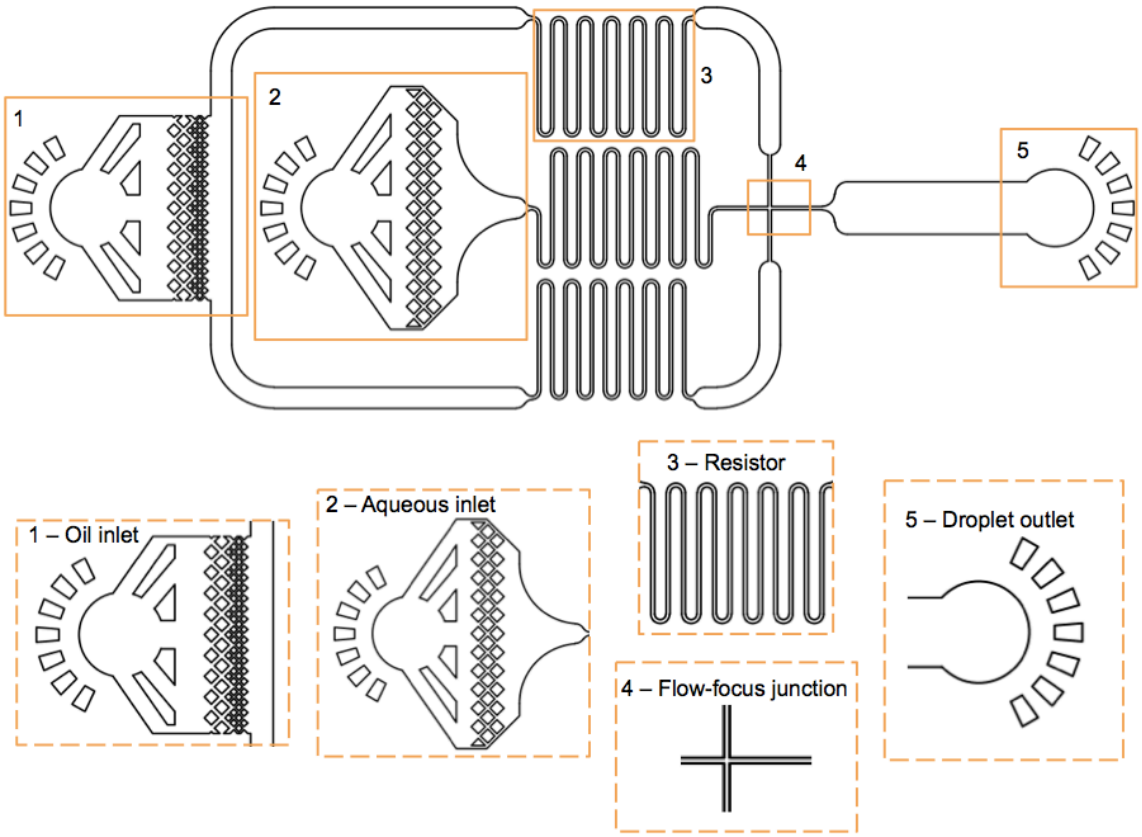

Figure 1 | Microfluidic device schematic. This pattern was used to generate microfluidic devices for the encapsulation of both liquid and agarose aqueous phases into a continuous oil phase. Fluids are pumped from syringes through tubing into the oil inlet (1) and the aqueous inlet (2). Fluid resistors (3) prevent rapid fluctuations in flow rate downstream in the device. Aqueous-in-oil droplets are produced at the flow-focus junction (4) through squeezing of the aqueous flow by the oil flow into a dispersed phase. Finally aqueous-in-oil droplets are collected through the droplet outlet (5) through a tube into a collection device. The device pattern was designed using autoCAD software.

Name	Sequence (5'->3')
Bio-TEG_P1_M6_MmeI_Short	/5BiotinTEG/GGGGACTTATCATCCAACCTGT
P1-M6-GAT-MmeI	CAAGCAGAAGACGGCATACGAGATAGACCGGGGACTTATCATCCAACCTGT
P2-ADPT-Tnseq-primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
ADBC-F-INDX1a	ACACGACGCTCTTCCGATCTGGAACTCCTACTGACNN
ADBC-F-INDX1b	ACACGACGCTCTTCCGATCTTAGCACATGCTGACTNN
ADBC-F-INDX1c	ACACGACGCTCTTCCGATCTCCTTGAGGATGACTGNN
ADBC-F-INDX1d	ACACGACGCTCTTCCGATCTATCGTGTACGACTGANN
ADBC-F-INDX2a	ACACGACGCTCTTCCGATCTGACTACCCTCTGGACNN
ADBC-F-INDX2b	ACACGACGCTCTTCCGATCTTCTGGATACACTACTNN
ADBC-F-INDX2c	ACACGACGCTCTTCCGATCTCTGATGGGAGACCTGNN
ADBC-F-INDX2d	ACACGACGCTCTTCCGATCTAGACCTATGTGATGANN
ADBC-F-INDX3a	ACACGACGCTCTTCCGATCTGACTGCCACATGAACNN
ADBC-F-INDX3b	ACACGACGCTCTTCCGATCTTCTGAATCTCCTGCTNN
ADBC-F-INDX3c	ACACGACGCTCTTCCGATCTAGAACGGTGTACTTGNN
ADBC-F-INDX3d	ACACGACGCTCTTCCGATCTCTGCTTAGAGGACGANN
ADBC-F-INDX4a	ACACGACGCTCTTCCGATCTCGACACTGAACGACCNN
ADBC-F-INDX4b	ACACGACGCTCTTCCGATCTTTCTGACTCCTTGATNN
ADBC-F-INDX4c	ACACGACGCTCTTCCGATCTGCTGTGACTTGCTGGNN
ADBC-F-INDX4d	ACACGACGCTCTTCCGATCTAAGACTGAGGAACTANN
ADBC-F-INDX5a	ACACGACGCTCTTCCGATCTACTGCATGCCTTAACNN
ADBC-F-INDX5b	ACACGACGCTCTTCCGATCTCTGAAGGAAACGGCTNN
ADBC-F-INDX5c	ACACGACGCTCTTCCGATCTTGACGTACGGAATTGNN
ADBC-F-INDX5d	ACACGACGCTCTTCCGATCTGACTTCCTTTGCCGANN
ADBC-F-INDX6a	ACACGACGCTCTTCCGATCTCCTAAAGGAATGGCCNN
ADBC-F-INDX6b	ACACGACGCTCTTCCGATCTATGGCGTACCCTAATNN
ADBC-F-INDX6c	ACACGACGCTCTTCCGATCTGGATTTCCTTACCGGNN
ADBC-F-INDX6d	ACACGACGCTCTTCCGATCTTACCGCATGGGATTANN
ADBC-F-INDX7a	ACACGACGCTCTTCCGATCTCAGACGAGGATTGCTNN
ADBC-F-INDX7b	ACACGACGCTCTTCCGATCTACACTTGTACCGAACNN
ADBC-F-INDX7c	ACACGACGCTCTTCCGATCTGTCTGCTCCTAACGANN
ADBC-F-INDX7d	ACACGACGCTCTTCCGATCTTGTGAACATGGCTTGNN
ADBC-F-INDX7a	ACACGACGCTCTTCCGATCTCAGACGAGGATTGCTNN
ADBC-F-INDX7b	ACACGACGCTCTTCCGATCTACACTTGTACCGAACNN
ADBC-F-INDX7c	ACACGACGCTCTTCCGATCTGTCTGCTCCTAACGANN
ADBC-F-INDX7d	ACACGACGCTCTTCCGATCTTGTGAACATGGCTTGNN
ADBC-F-INDX8a	ACACGACGCTCTTCCGATCTACGCGAGGACCTACCNN
ADBC-F-INDX8b	ACACGACGCTCTTCCGATCTCAATTCTAGATGGATNN
ADBC-F-INDX8c	ACACGACGCTCTTCCGATCTTGCGCTCCTGGATGGNN
ADBC-F-INDX8d	ACACGACGCTCTTCCGATCTGTTAAGATCTACCTANN
ADBC-F-INDX9a	ACACGACGCTCTTCCGATCTCTCAGAAGACCGGATNN
ADBC-F-INDX9b	ACACGACGCTCTTCCGATCTAGTGTGCACATTACCNN
ADBC-F-INDX9c	ACACGACGCTCTTCCGATCTGAGTCTTCTGGCCTANN
ADBC-F-INDX9d	ACACGACGCTCTTCCGATCTTCACACGTGTAATGGNN
ADBC-F-INDX10a	ACACGACGCTCTTCCGATCTACCGTACGTATTAATNN
ADBC-F-INDX10b	ACACGACGCTCTTCCGATCTCTAAGGAAGCCGGCCNN
ADBC-F-INDX10c	ACACGACGCTCTTCCGATCTTGGCATGCATAATTANN
ADBC-F-INDX10d	ACACGACGCTCTTCCGATCTGATTCCTTCGGCCGGNN
ADBC-F-INDX11a	ACACGACGCTCTTCCGATCTCACTCGGACACGAATNN
ADBC-F-INDX11b	ACACGACGCTCTTCCGATCTTCTGATACTCTTGCCNN
ADBC-F-INDX11c	ACACGACGCTCTTCCGATCTGGGAGACTATGCTTANN
ADBC-F-INDX11d	ACACGACGCTCTTCCGATCTATACTCTGGGAACGGNN
ADBC-F-INDX12a	ACACGACGCTCTTCCGATCTCCATACGAGCACTCCNN
ADBC-F-INDX12b	ACACGACGCTCTTCCGATCTTTCCGATGTTGAGTANN
ADBC-F-INDX12c	ACACGACGCTCTTCCGATCTGGTATGCTCGTGAGGNN
ADBC-F-INDX12d	ACACGACGCTCTTCCGATCTAAGGCTACAACTCATNN

Table 1 | List of primers used in the study.

Name	Sequence(5'->3')
ADBC-R-INDX1a	/5PHOS/GTCAGTAGGAGTTCCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX1b	/5PHOS/AGTCAGCATGTGCTAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX1c	/5PHOS/CAGTCATCCTCAAGGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX1d	/5PHOS/TCAGTCGTACACGATAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX2a	/5PHOS/GTCCAGAGGGTAGTCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX2b	/5PHOS/AGTAGTGTATCCAGAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX2c	/5PHOS/CAGGTCTCCCATCAGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX2d	/5PHOS/TCATCACATAGGTCTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX3a	/5PHOS/GTTCATGTGGCAGTCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX3b	/5PHOS/AGCAGGAGATTCAGAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX3c	/5PHOS/CAAGTACACCGTTCTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX3d	/5PHOS/TCGTCCTCTAAGCAGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX4a	/5PHOS/GGTCGTTCAGTGTCGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX4b	/5PHOS/ATCAAGGAGTCAGAAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX4c	/5PHOS/CCAGCAAGTCACAGCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX4d	/5PHOS/TAGTTCCTCAGTCTTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX5a	/5PHOS/GTTAAGGCATGCAGTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX5b	/5PHOS/AGCCGTTTCCTTCAGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX5c	/5PHOS/CAATTCCGTACGTCAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX5d	/5PHOS/TCGGCAAAGGAAGTCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX6a	/5PHOS/GGCCATTCCTTTAGGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX6b	/5PHOS/ATTAGGGTACGCCATAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX6c	/5PHOS/CCGGTAAGGAAATCCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX6d	/5PHOS/TAATCCCATGCGGTAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX7a	/5PHOS/AGCAATCCTCGTCTGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX7b	/5PHOS/GTTCGGTACAAGTGTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX7c	/5PHOS/TCGTTAGGAGCAGACAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX7d	/5PHOS/CAAGCCATGTTCACAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS/
ADBC-R-INDX7a	5PHOS/AGCAATCCTCGTCTGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX7b	5PHOS/GTTCGGTACAAGTGTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX7c	5PHOS/TCGTTAGGAGCAGACAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX7d	5PHOS/CAAGCCATGTTCACAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX8a	5PHOS/GGTAGGTCCTCGCGTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX8b	5PHOS/ATCCATCTAGAATTGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX8c	5PHOS/CCATCCAGGAGCGCAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX8d	5PHOS/TAGGTAGATCTTAACAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX9a	5PHOS/ATCCGGTCTTCTGAGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX9b	5PHOS/GGTAATGTGCACACTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX9c	5PHOS/TAGGCCAGAAGACTCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX9d	5PHOS/CCATTACACGTGTGAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX10a	5PHOS/ATTAATACGTACGGTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX10b	5PHOS/GGCCGGCTTCCTTAGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX10c	5PHOS/TAATTATGCATGCCAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX10d	5PHOS/CCGGCCGAAGGAATCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX11a	5PHOS/ATTCGTGTCCGAGTGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX11b	5PHOS/GGCAAGAGTATCAGAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX11c	5PHOS/TAAGCATAGTCTCCCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX11d	5PHOS/CCGTTCCCAGAGTATAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX12a	5PHOS/GGAGTGCTCGTATGGAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX12b	5PHOS/TACTCAACATCGGAAAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX12c	5PHOS/CCTCACGAGCATACCAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS
ADBC-R-INDX12d	5PHOS/ATGAGTTGTAGCCTTAGATCGGAAGAGCGTCGTGTAGGGA/3PHOS

Table 1 continued | List of primers used in the study.

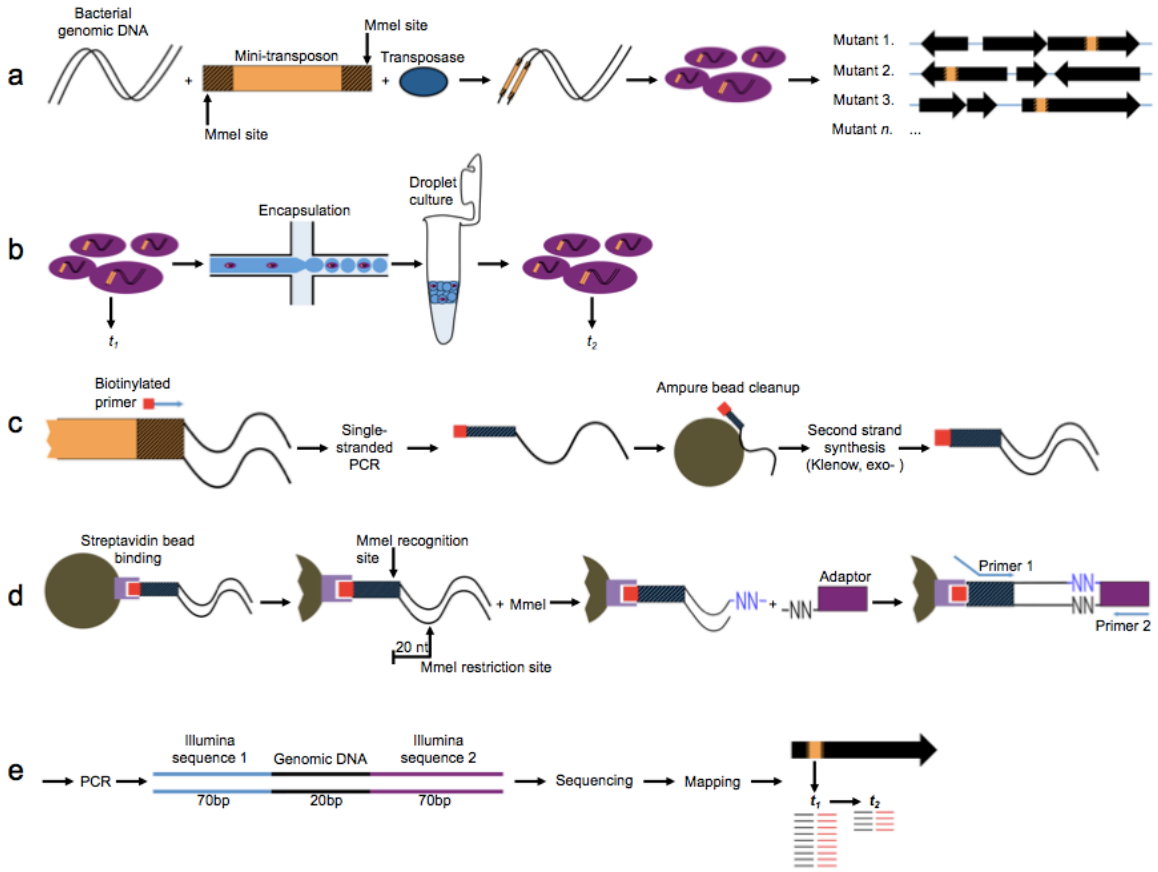

Figure 2 | Schematic of Tn-seq performed utilizing a droplet-based technique. (a) A Mariner mini-transposon that contains MmeI sites in the inverted repeat ends is first transposed into genomic DNA *in vitro* and then transformed into a bacterial population. Each resulting mutant contains one insertion. (b) DNA is isolated from one portion of the cultured transposon mutant pool (t_1) while another portion is used for growth selection in droplets, after which DNA is collected from these encapsulated cells (t_2). (c) To create a DNA sequencing library first a biotinylated primer is used to enrich the transposon ends using single stranded PCR. Ampure beads are used to clean the sample from excess unused primer. Second strand synthesis is then performed using Klenow fragment. (d) Biotinylated double-stranded products are then bound to streptavidin beads. On-bead enzymatic reactions are performed that include MmeI digestion, Alkaline phosphatase incubation, and adaptor ligation. (e) On-bead PCR amplification is followed by Illumina sequencing and mapping. Samples are identified by barcode sequence from the adaptors, and read counts on either side of each mapped transposon insertion allow for fitness calculation.

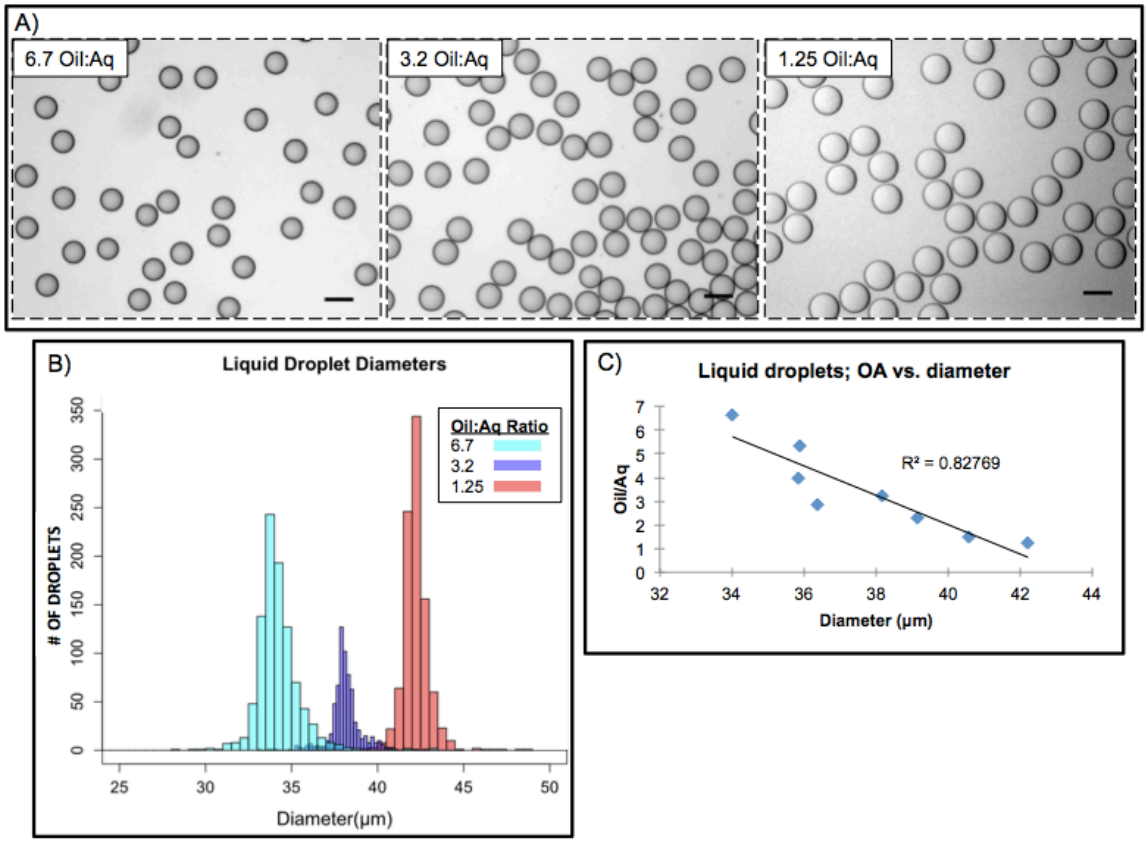

Figure 3 | Liquid droplet characteristics. (A) Differential interference contrast (DIC) images of liquid droplets at three different oil/aqueous (Oil:Aq) flow ratios. Scale bar = 40µm (B) Distribution of droplet diameters at three different OA flow ratios. (C) Correlation plot of mean diameter of droplets produced from different OA flow ratios.

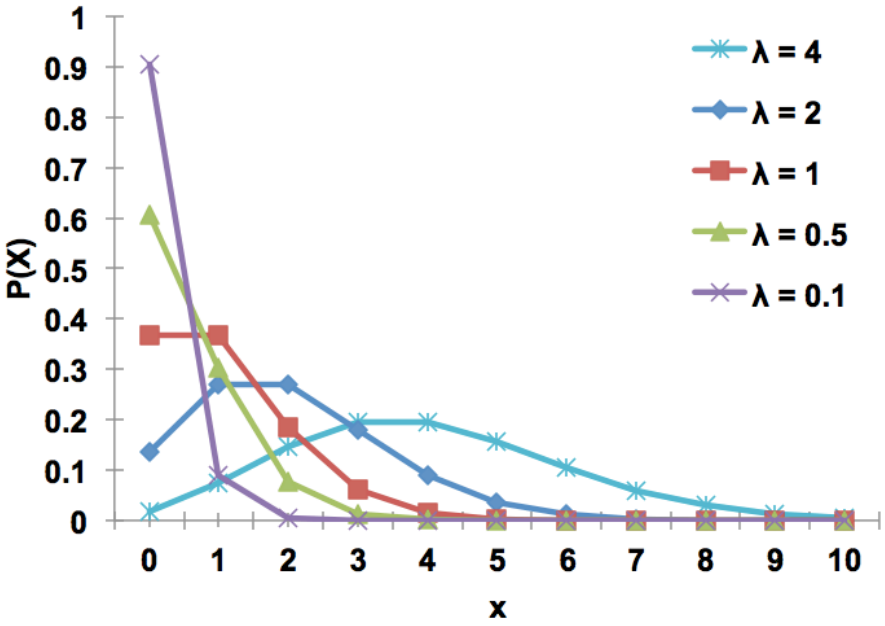

Figure 4 | Poisson distribution showing the probability [P(x)] of encapsulating x number of cells within a droplet at different average number of cells per volume (λ)

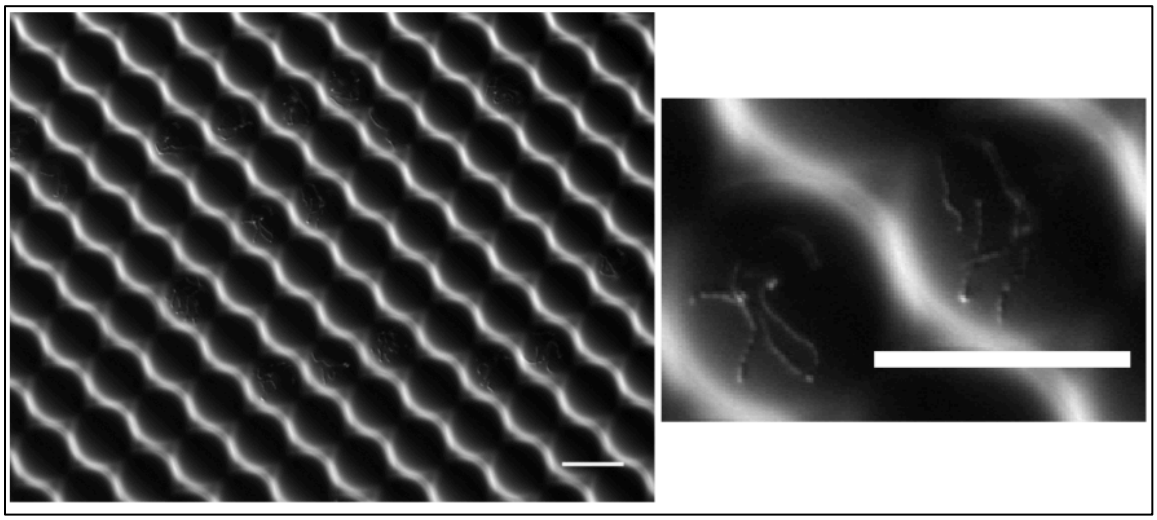

Figure 5 | Differential interference contrast (DIC) image of *S. pneumoniae* strain D39 after 5hrs culture in liquid droplets. Images were used to count empty droplets to predict cell occupancy based on a Poisson distribution. Scale bar = 40μm.

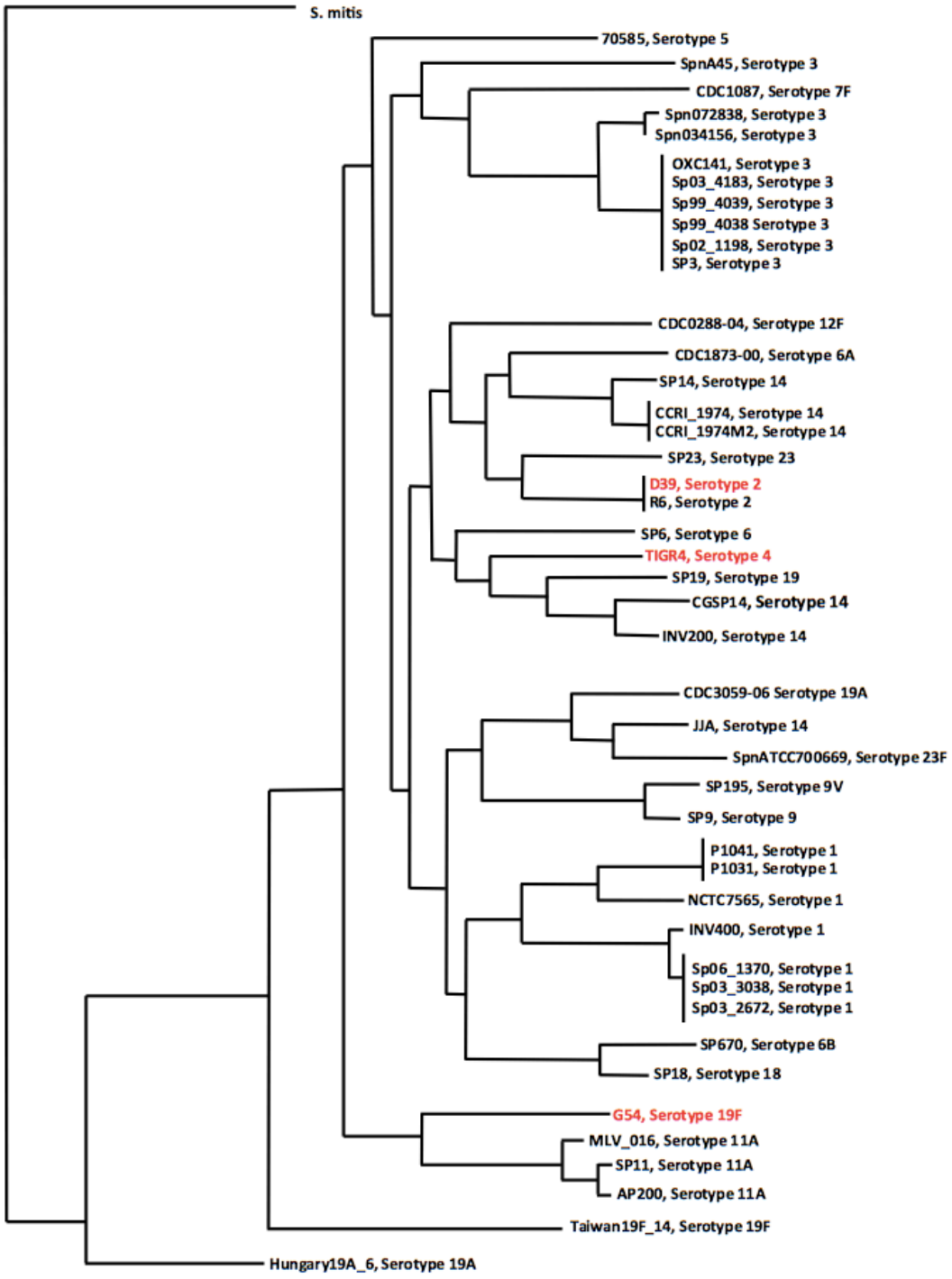

Figure 6 | Phylogenetic tree including *S. pneumoniae* strains used in this study (highlighted in red).

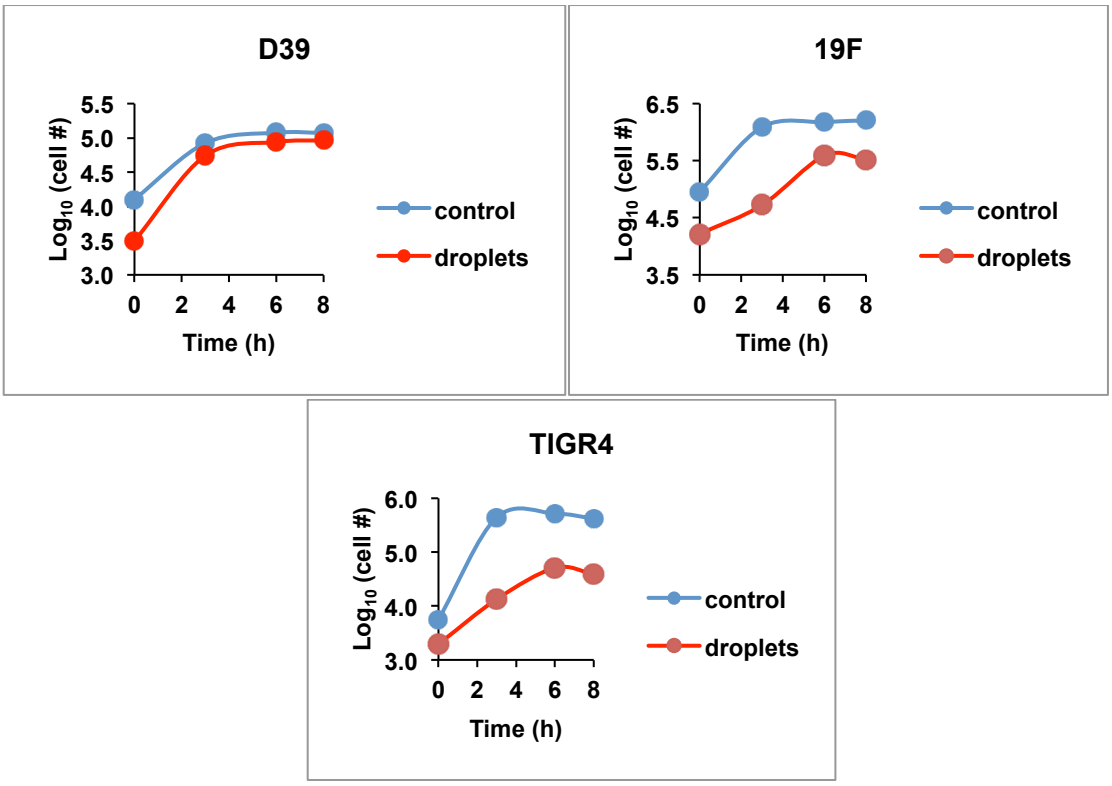

Figure 7 | Growth profiles of 3 different *S. pneumoniae* strains in droplets and bulk culture (control) over an 8hr time period. Strains were cultured in semi-defined minimal media (SDMM) and plated on blood agar at different time points to determine live cell counts.

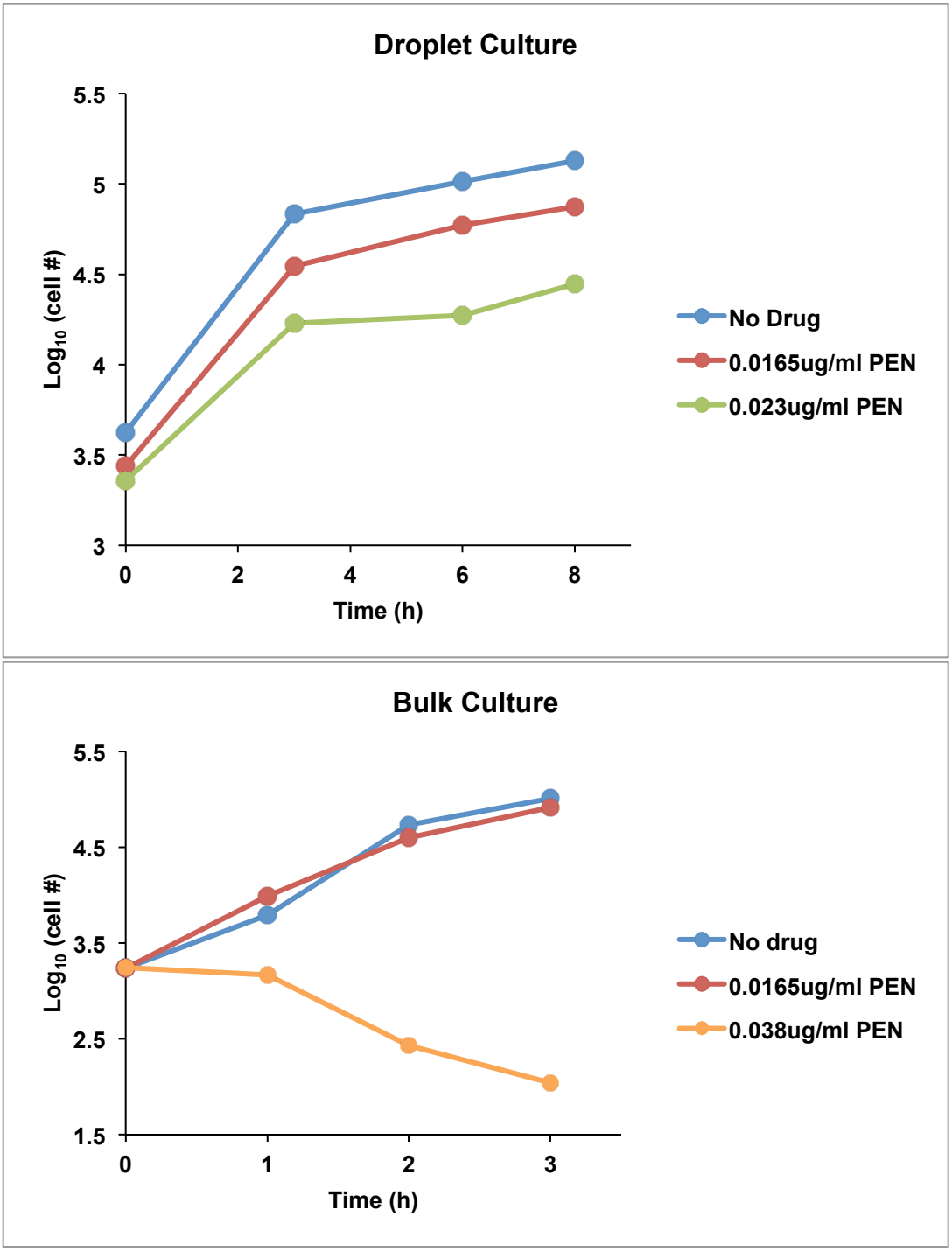

Figure 8 | Growth profiles of *S. pneumoniae* strain TIGR4 cultured in droplets and bulk culture and treated in the absence (No drug) and presence of Penicillin (PEN). Strains were cultured in semi-defined minimal media (SDMM) and plated on blood agar at different time points to determine live cell counts.

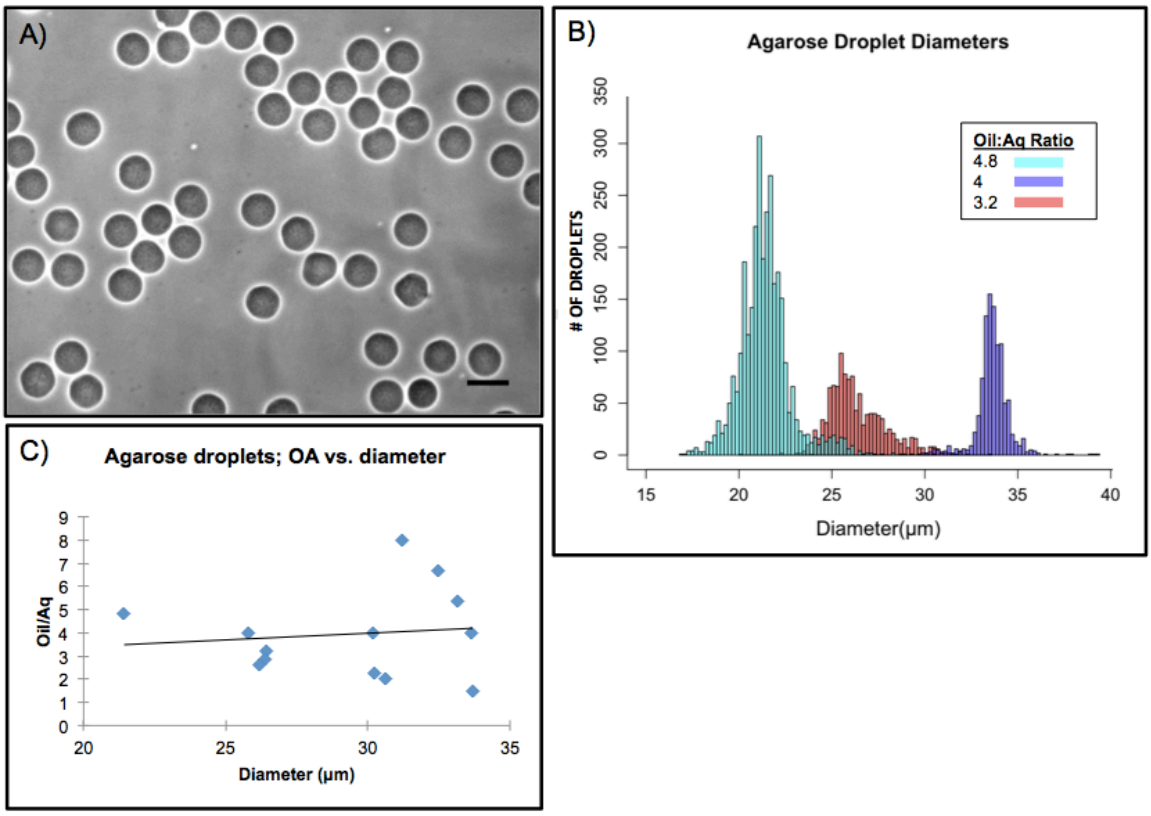

Figure 9 | Agarose droplet characteristics. (A) Phase contrast image of agarose droplets. Scale bar = 40μm (B) Distribution of droplet diameters at three different oil:aqueous (OA) flow ratios. (C) Correlation plot of mean diameter of droplets produced from different OA flow ratios.

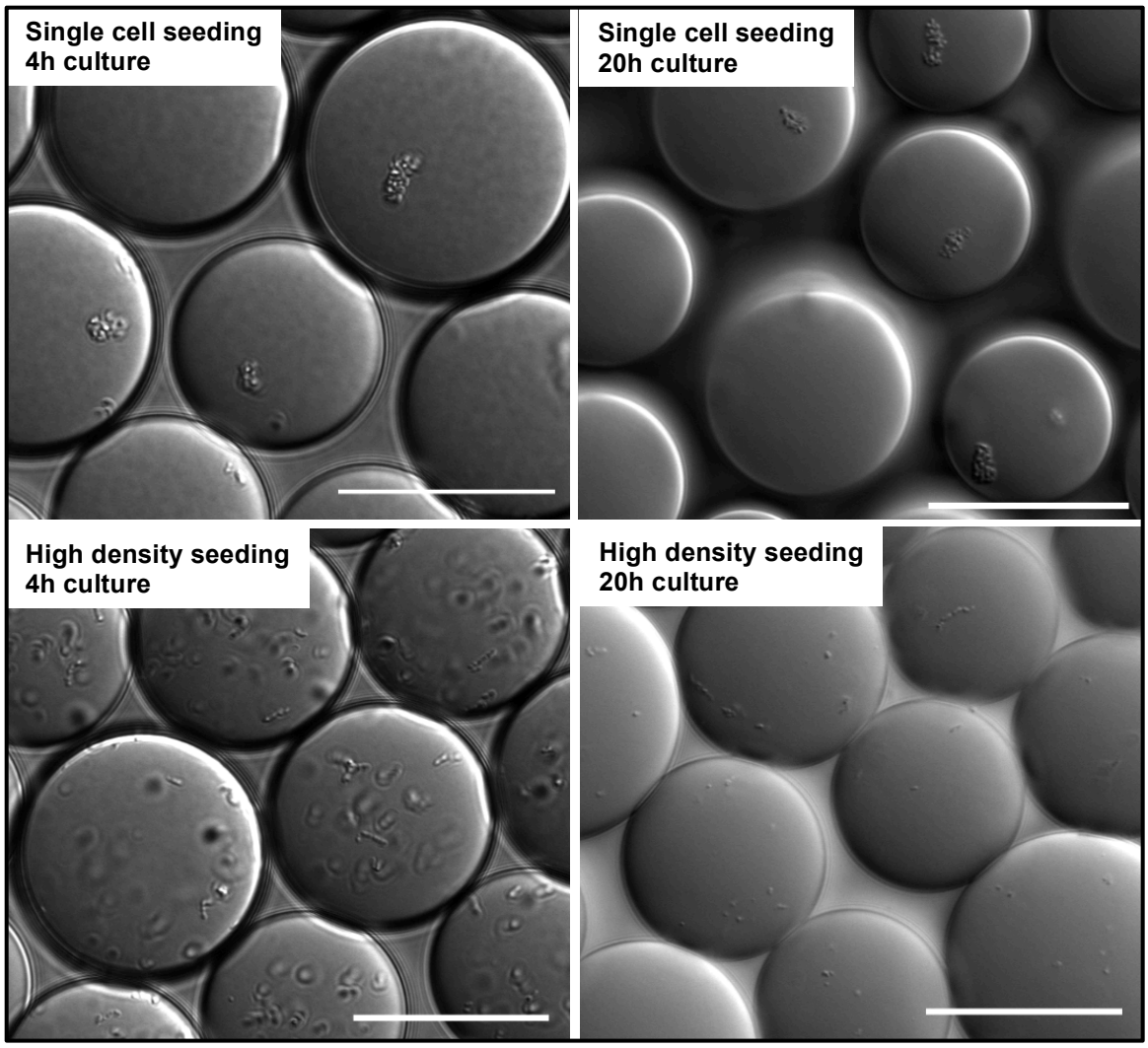

Figure 10 | *S. pneumoniae* strain D39 was seeded in 1.5% agarose droplets at either single cell or high density. Droplets were cultured at 37°C, 5% CO_2, and DIC images of representative droplets were taken at 4hrs and 20hrs. Microcolonies approximately 10μm in size were produced after 4hrs in single cell seeding while microcolony formation was not observed in droplets seeded in a high density after 4hrs. After 20hrs of growth microcolonies that were produced in droplets seeded with single cells remained relatively intact while cells in the high-density droplets looked like they had undergone cell death. Scale bar = 40μm.

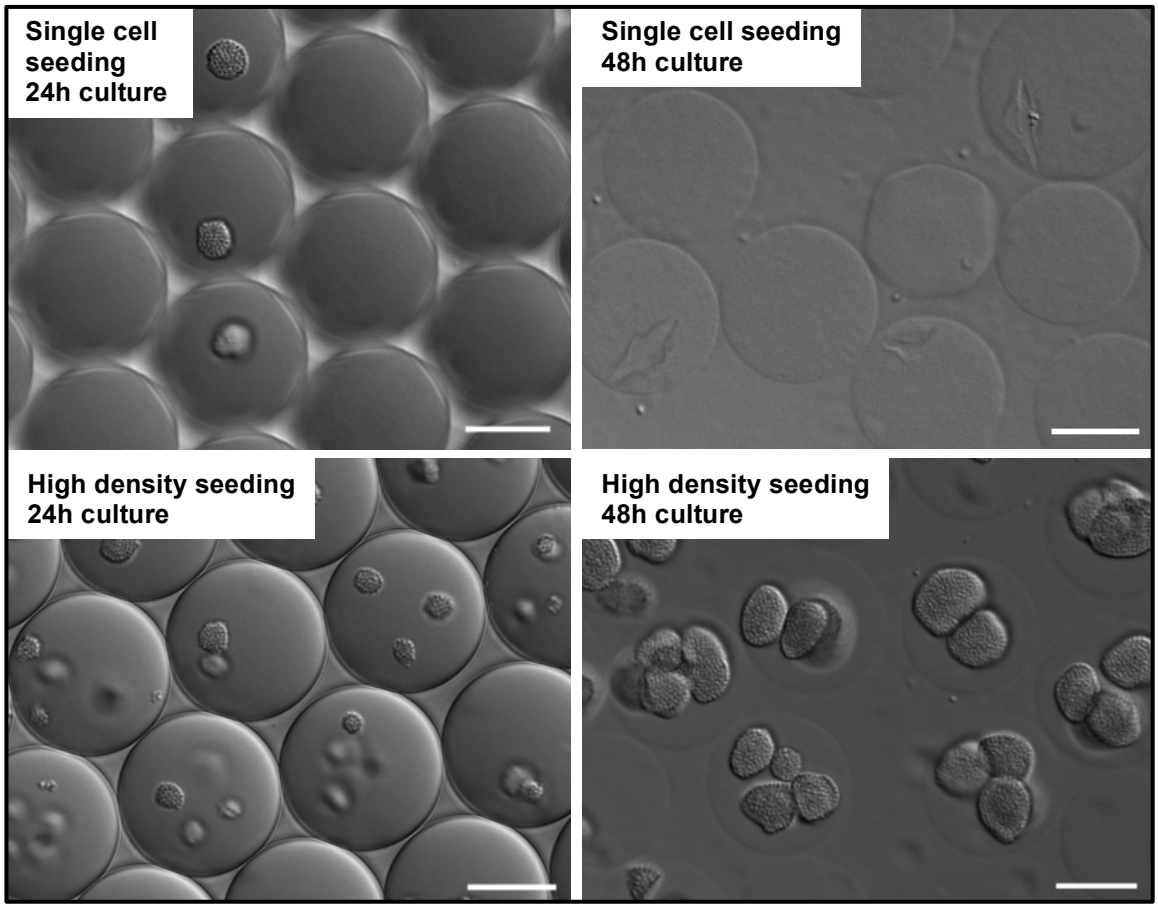

Figure 11 | *A. baumannii* was seeded in 1.5% agarose droplets at either single cell or high density. Cells were culture in oil droplets at 37°C for 24hrs, at which time representative DIC images were taken. Oil was removed using PFO and intact droplets were re-submerged in new growth media. At 48hrs total growth DIC images of representative droplets were taken. After 24hrs healthy microcolonies develop in both single cell and high density seeding conditions, and active proliferation can be re-established by removing the oil layer and supplying fresh media. Additionally we observe a density dependent "escape" phenotype when cell densities are at single cell seeding levels as opposed to high density seeding. Scale bar = 40μm.

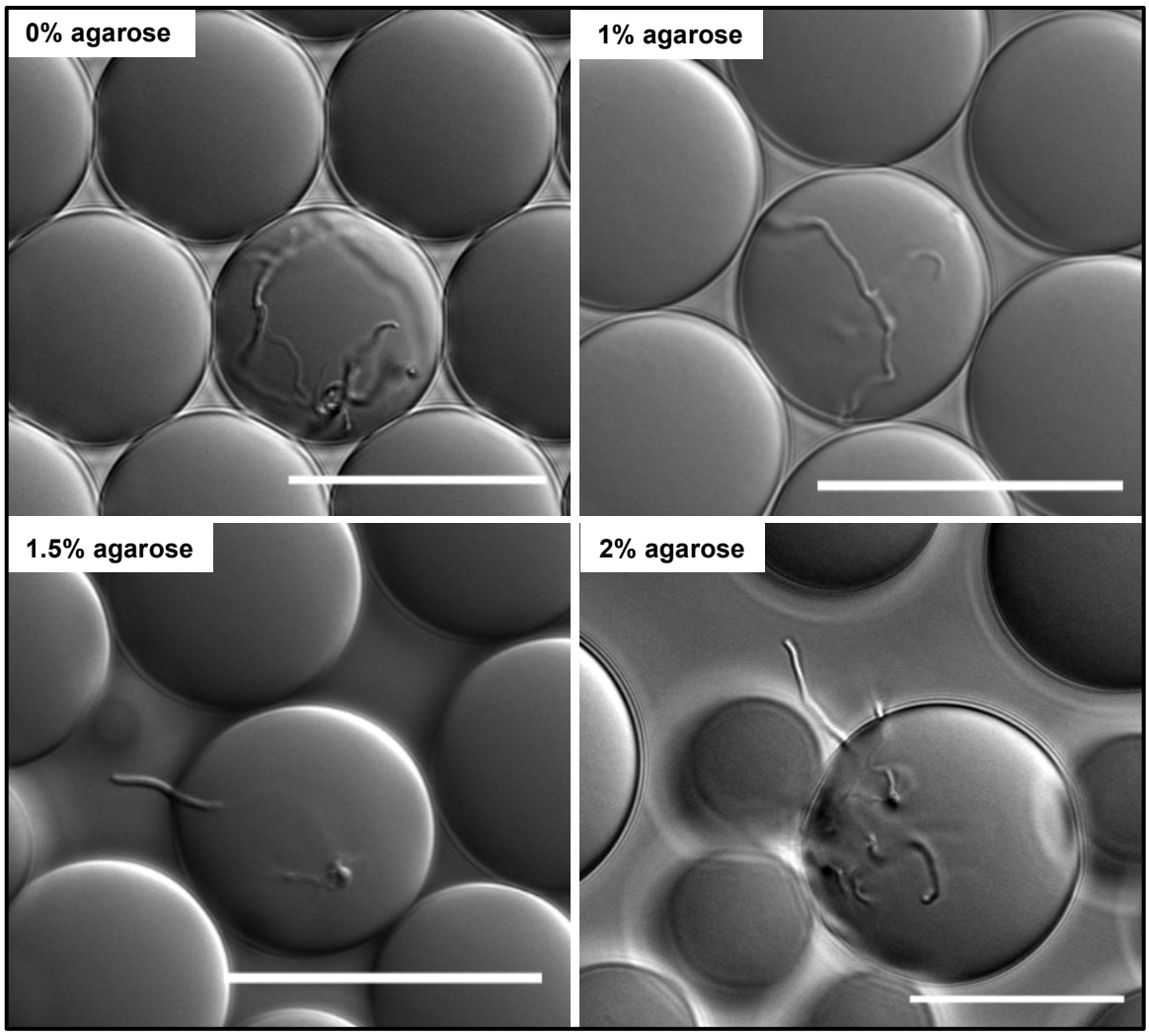

Figure 12 | *S. roseosporus* spores were seeded in droplets containing different concentrations of agarose (0%, 1%, 1.5%, and 2%). Droplets surrounded in oil were cultured at 30°C for 48hrs after which representative DIC images were taken. It seems that *S. roseosporus* can push better off of denser agarose matrix and can use the apical end of a single filament to penetrate through the oil. Scale bar = 40μm.

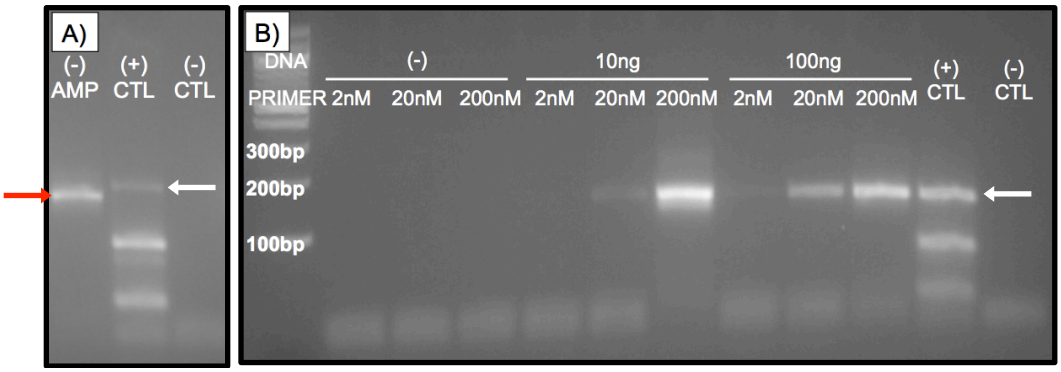

Figure 13 | PCR results of new low-input DNA library preparation. (A) Without SPRI bead clean up a product that is approximately 20bp smaller (red arrow) than the correct library size (white arrow) is produced. (B) DNA libraries produced in the absence (-) or presence of indicated amounts of DNA. Final biotinylated primer concentrations used for single stranded PCR enrichment are shown. SPRI bead cleanup is used for all conditions. The incorrect PCR product size due to direct primer-adaptor binding is prevented by SPRI bead clean up (-DNA), while a band matching the correct library size was produced under the same conditions in the presence of 10ng and 100ng of genomic DNA. A library generated using the traditional Tn-seq preparation method is shown (+CTL). The use of Ampure beads and the concentration of biotinylated primer are critical components for producing an Illumina library of DNA molecules of the correct size.

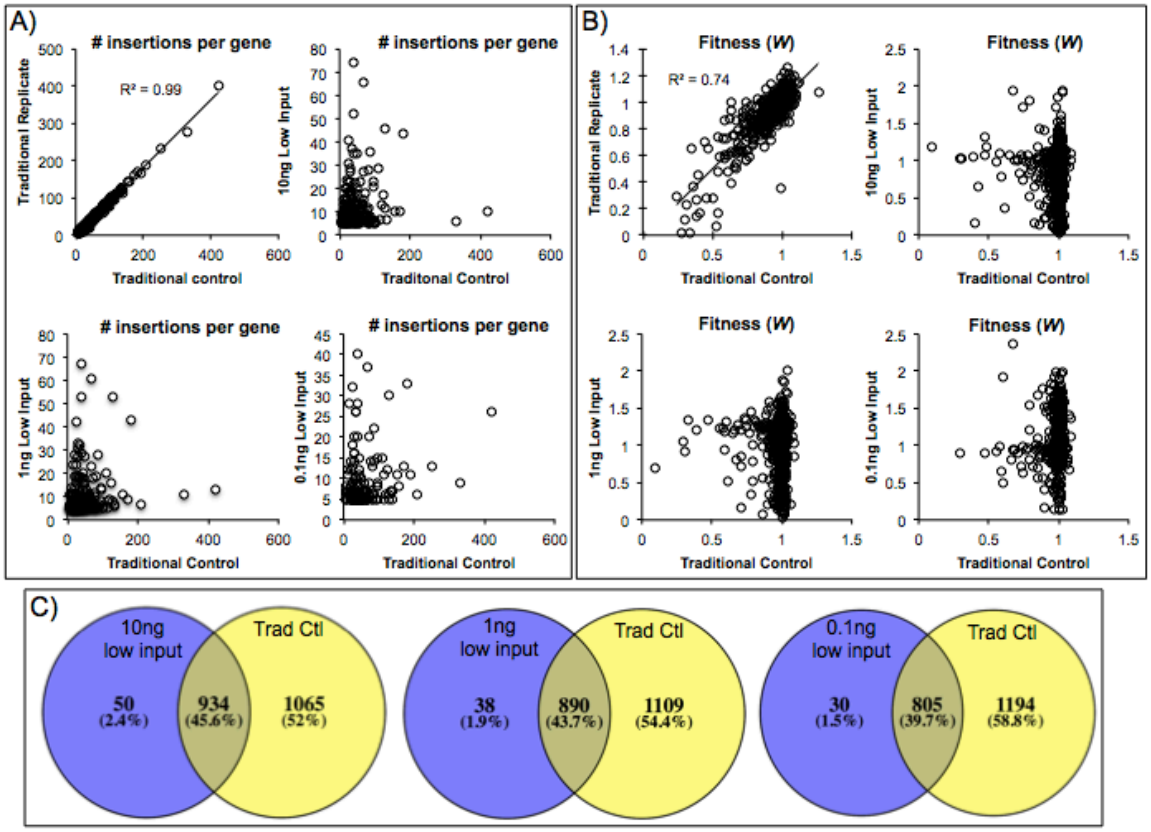

Figure 14 | Data comparing number of insertions per gene and fitness (*W*) between a new low input Tn-seq DNA library preparation to the traditional library preparation. Data from new preparations does not correlate to traditional library preparations because of a low diversity of reads obtained from the new preparation. (A) The number of insertions per gene from a traditional Tn-seq preparation (Traditional Control) is correlated to a biological replicate (Traditional Replicate) and to three low input library preparations (10ng, 1ng, and 0.1ng). Only genes that had greater than 5 insertions were plotted. (B) Fitness values for a traditional Tn-seq preparation (Traditional Control) are correlated to a biological replicate (Traditional Replicate) and to all three low input library preparations (10ng, 1ng, and 0.1ng). Gene fitness was only plotted if data was obtained for that gene in both data sets. (C) Each set of genes that contained at least 1 insertion where compared between the low input preparations and the traditional preparation (Trad Ctl).

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